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Inhalation Toxicologic Procedures for Exposure of Small Laboratory Animals to Highly Toxic Materials:

Part B: Exposures to Aerosols with Notes on Toxicity of Ricin

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INHALATION TOXICOLOGIC PROCEDURES FOR EXPOSURE OF SMALL LABORATORY ANIMALS TO HIGHLY TOXIC MATERIALS: PART B: EXPOSURES TO AEROSOLS WITH NOTES ON TOXICITY OF RICIN

by

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Abstract

The estimation of military hazard from toxic materials usually requires an estimate of the human toxicity. With expensive, rare and highly toxic substances, only small amounts of substance may be available for testing or safety considerations may restrict the amounts of material that may be used at one time. A functional modular system has been developed for the exposure of mice, rats and guinea pigs to highly toxic liquid, gas or aerosol test materials that requires a minimum of trial material.

The total system is described in this aerosol centred report and in companion reports which describe methods for use with gasses. The aerosol exposure system is operated dynamically in a chamber with a very small volume. Provision is made to continuously monitor the concentration and composition of the aerosol in the test atmospheres and grab samples are taken to establish the exposure concentrations. Respiration of the subjects may be continuously monitored during the exposures but because of the restraint of the animals required by the nose only configuration, observations of behaviour during the exposures are not possible.

The development and validation of this configuration are described and toxicity studies with Ricin are used as examples. The observed nose only inhalation toxicity of Ricin to rats was 4 mg.min/m^3. Times-to-death were generally 24 - 48 hr following significant weight loss. Toxicity values from other laboratories range from 2 to 4 mg.min/m^3.

Resumé

L'évaluation des dangers que posent les matières toxiques pour les militaires nécessite habituellement une estimation de la toxicité pour les humains. Avec des substances rares, très toxiques et chères, on ne dispose que de faibles quantités pour les essais, ou des considérations d'ordre sécuritaire peuvent limiter les quantités de matières qui peuvent être utilisées en une fois. On a mis au point un système modulaire fonctionnel pour l'exposition des souris, des rats et des cobayes aux matières d'essai liquides, gazeuses ou en aérosols très toxiques, système qui nécessite un minimum de matériel d'essai.

L'ensemble du système est décrit dans ce rapport centré sur les aérosols et dans des rapports complémentaires qui décrivent les méthodes utilisées avec les gaz. Le système d'exposition aux aérosols est exploité de façon dynamique, dans un compartiment de très petit volume. On peut surveiller en continu la concentration et la composition de l'aérosol dans les atmosphères d'essai et on prélève des échantillons ponctuels pour établir les concentrations d'exposition. La respiration des sujets peut être surveillée en continu durant les expositions, mais il a été impossible d'observer leur comportement durant les expositions étant donné que les animaux avaient un appareil de respiration fixé au nez.

On décrit la mise au point et la validation de cette configuration, et les études de toxicité faites avec le Ricin servent d'exemples. La toxicité au Ricin lors de l'inhalation par le nez seulement observée sur les rats était de 4 mg.min/m^3. Les sujets mouraient habituellement 24 à 48 heures (temps de survie) après une importante perte de poids. Les valeurs de la toxicité provenant d'autres laboratoires variaient de 2 à 4 mg.min/m^3.

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Executive summary

Background

Estimation of the toxicity of inhaled vapours and aerosols and an estimate of the toxicity to man are basic parts of military hazard assessment. For military purposes, the estimates required are usually for highly toxic materials and, complicating the issue, materials that often are in very short supply. An exposure system was required that would provide inhalation toxicology values for very toxic materials, using small volume chambers both for safety and thrift reasons, that would be flexible enough to handle both gasses and aerosols and provide continuous recordings of respiration and chamber concentration. What has been developed is a modular exposure system that provides for these needs, is readily and relatively inexpensively made, can be quickly decontaminated, will allow use of a variety of research animal species and, by various rearrangements, will operate with gasses or aerosols in both recirculating and dynamic modes. The system has been in use for several years at Defence Research Establishment Suffield for a wide variety of studies but has never been described in detail in a general forum that could be distributed openly. Operation of the system with air dissolved chemical is described in Part A. This paper, Part B, describes the configuration used with toxic aerosols and the operational capabilities, parameters and limitations.

Capabilities of the system

The modular aerosol system is used for the estimation of inhalation toxicity of aerosols (expressed as the concentration - time product or LCt_{50}) in a variety of laboratory species, while using minimal amounts of test material (*eg.* 4 mg protein or soluble solid, 50 μL liquid). Mice, rats and guinea pigs are readily accommodated. The aerosol exposure system operates in the dynamic mode. Continuous records are created of the particle sizes, numbers and concentrations in the test atmosphere which are used to monitor the operation of the system. However, because the particle measurement instruments do not function well with particles less than 0.5 μm diameter, the concentration of trial substance is calculated from grab samples. The respiration of the animals can be followed during the exposures. The components are made of Plexiglas® so that cleaning and decontamination are readily accomplished or, in the worst case, destroyed without major loss. Exposures of 10 - 30 min are readily performed and longer exposures are possible with due care for the welfare of the test subjects.

Ricin toxicity

The nose only inhalation toxicity of ricin aerosol to rats, described as an example of the operation of the system, was estimated to be 4 mg.min/m^3. Times -to-death were 24 - 48 hr. Other laboratories have reported ricin toxicity values between 2 and 4 mg.min/m^3.

Bide, R.W., Risk, D.J., Ladouceur, M.F. and Beifus, D.G. (2000). Inhalation toxicologic procedures for exposure of small laboratory animals to highly toxic materials; Part B: Exposures to aerosols with notes on the toxicity of Ricin (U). (DRES TM 2000-066). Defence Research Establishment Suffield. UNCLASSIFIED.

Sommaire

Contexte

L'estimation de la toxicité des vapeurs et des aérosols inhalés et une estimation de la toxicité pour les humains sont les principales composantes de l'évaluation des dangers pour les militaires. À des fins militaires, les estimations nécessaires se font habituellement sur des matières très toxiques et, ce qui complique les choses, il s'agit de matières rarement disponibles en grande quantité. Il fallait donc un système d'évaluation de l'exposition qui donnerait des valeurs de toxicologie de l'inhalation pour des matières très toxiques, dans des compartiments de faible volume, tant pour des raisons de sécurité que d'économie, qui serait assez souple pour traiter à la fois les gaz et les aérosols tout en fournissant des données en continu sur la respiration et la concentration dans le compartiment. On a donc mis au point un système d'exposition modulaire qui répond à ces besoins, qui est facile à utiliser et qui coûte relativement peu cher, qui est facile à décontaminer, qui permet d'utiliser diverses espèces d'animaux de recherche et, après divers réarrangements, fonctionnera avec des gaz ou des aérosols à la fois en mode de recirculation et en mode dynamique. Le système a été utilisé pendant plusieurs années au Centre de recherche pour la défense Suffield pour une grande variété d'études, mais il n'a jamais été décrit en détail dans un format qui puisse être distribué ouvertement. Dans la partie A, on décrit le fonctionnement du système avec des substances chimiques dissoutes dans l'air. Ce rapport, Partie B, décrit la configuration utilisée avec des aérosols toxiques et les capacités, limites et paramètres opérationnels.

Capacités du système

Les système d'aérosol modulaire est employé pour l'évaluation de la toxicité de l'inhalation des aérosols (exprimée en produit de la concentration et du temps ou CLt_{50}) chez diverses espèces d'animaux de laboratoire, tout en utilisant des quantités minimes de matière d'essai (p. ex. 4 mg de protéine ou de solide soluble, 50 µL de liquide). Le système fonctionne bien avec les souris, les rats et les cobayes. Le système d'exposition aux aérosols fonctionne en mode dynamique. On crée des données en continu sur la taille, le nombre et la concentration des particules dans l'atmosphère d'essai, qui sont ensuite utilisées pour surveiller le fonctionnement du système. Toutefois, étant donné que les instruments de mesure des particules ne fonctionnent pas bien avec des particules d'un diamètre inférieur à 0,5 µm, la concentration de la substance d'essai est calculée sur des échantillons ponctuels. La respiration des animaux peut être suivie durant les expositions. Les composantes sont faites en Plexiglas®, ce qui facilite le nettoyage et la décontamination ou, dans le pire cas, permet de détruire l'appareil sans occasionner des pertes majeures. Les expositions de 10 à 30 min sont faciles à faire, et des expositions plus longues sont possibles avec diligence normale pour le bien-être des sujets d'essais.

Toxicité du Ricin

La toxicité des aérosols de Ricin inhalés par le nez seulement chez les rats, décrite comme un exemple du fonctionnement du système, a été estimée à 4 mg.min/m³. Les temps de survie étaient de 24 à 48 heures. D'autres laboratoires ont signalé des valeurs de toxicité du Ricin allant de 2 à 4 mg.min/m³.

Bide, R.W., Risk, D.J., Ladouceur, M.F. and Beifus, D.G. (2000). Inhalation toxicologic procedures for exposure of small laboratory animals to highly toxic materials; Part B: Exposures to aerosols with notes on the toxicity of Ricin (U). (DRES TM 2000-066). Defence Research Establishment Suffield. UNCLASSIFIED.

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DRES animal care statement

In conducting the research described in this report, the investigators adhered to the "Guide to the Care and Use of Experimental Animals" published by the Canadian Council on Animal Care.

Introduction

Inhalation toxicology is a basic part of military hazard assessment. Older techniques exposed relatively large numbers of animals "whole body" to air dissolved or aerosolized materials. Newer techniques expose animals "nose only" to directed streams of test materials. As currently practised in most laboratories, both methods tend to use relatively large amounts of test material (kg). In recent years, the use of experimental animals has come under more and more scrutiny and methods which use large numbers of animals have been severely criticised. Concomitant with these changes, the requirement has arisen to provide toxicologic data from small (mg) to minute (ug) amounts of test material, either because of cost, extremely limited availability of material or extreme toxicity. The result has been a search for safer and more efficient methods for toxicologic testing.

Assessment of the military hazard posed by inhaled substances requires estimation of the toxicity of materials inhaled by man. Estimations of the human exposure response are usually made by extrapolation from data describing the toxicity observed in a number of laboratory animal species [1, 2, 3, 4, 5, 6, 7]. Because of the expense, in both specie and manpower, associated with such studies, materials are often first screened for toxicity in mice and further testing is done only for those substances that prove sufficiently toxic or are of special interest for other reasons.

Normally, inhalation exposures are done in flow-through or "dynamic" chambers, systems which present a constant flow of "fresh" atmosphere to the animal subjects to minimize problems associated with O_2 depletion, build up of CO_2, NH_3 and other metabolites and changes in the test atmosphere caused by metabolism of the test chemical [1, 2, 3]. When only small amounts of test chemical are available, the entire exposure system must be miniaturised to be effective (Table 1). In addition, when highly toxic materials were to be assessed, there was a need for inhalation exposure systems that could be readily cleaned and decontaminated or, in the worst case, disposed of without undue cost or program disruption. These goals have been achieved with the modular system described in this and a companion report [8].

The modular system developed may be used for exposures either to gasses or aerosols with mice, rats, guinea pigs and ferrets. As the configuration described in this report was intended for aerosols, the recirculating and static options were not available. In the configuration chosen, the volume of the exposure chamber was one Liter to minimize both the amount of expensive test material required and the potential hazard to operators and the environment when handling extremely toxic materials.

The parts of the system are either readily obtainable on the open market or easily constructed from Plexiglas® by a machine or fabricating shop. The concentration and particle numbers in the test atmosphere can be continuously monitored and, as an added bonus, respiration monitors may be used with the system. A study of the inhalation toxicity of aerosolized Ricin is provided as an example of the use of this version of the system.

Table 1
Material required for one exposure at 1.0 mg/m³ for 10 min

System	μg
100 L dynamic chamber (1000 L/min flow)	10,000
6 L dynamic chamber (60 L/min flow)	600
1 L dynamic system (5 L/min flow)	50
12 L recirculating system	12
5 L static system	5

Materials and methods

Chemicals

Bovine serum albumin, fraction V salt free (BSA), bovine haemoglobin (Hb), ricin, universally labelled C^{14}-sucrose (360-720 mCi/mmol in a 9:1 ethanol:water), universally labelled C^{14}-glucose (250-360 mCi per mmol in water) and 1-C^{14}-iodoacetamide were purchased from Sigma Chemical Co. St. Louis, MO. Phosphate buffered isotonic saline (Dulbecco A) was prepared as a sterile solution (PBS) from a prepared commercial powder mixture (Oxoid Ltd., England).

C^{14}-labelled Hb was prepared by incubating 1 volume of 100 mg/mL Hb with labelled iodoacetamide (2.25 mol per mol Hb) for one hour in a PBS solution and then dialysing the mixture against 3 changes of 10 volumes of PBS. The radioactivity in the resulting solution was bound to the Hb. Five mL aliquots were frozen in glass tubes for storage and future use.

Animals

The male rats used in this study were outbred Crll:CD®(SD)BR strain purchased from Charles River Canada, St. Constant, Que. They were acclimatized for at least 7 days in the Vivarium at Defence Research Establishment Suffield (DRES) before use.

Air supply

Particle and chemical free air was supplied by a breathing air system consisting of a water sealed compressor (SIHI model KHPE 35504 SIHI-Halberg International Co., Ludwigshafen/ Rhein, West Germany) fitted with HEPA filters on the air intake, a water separator and air dryer immediately following the compressor and then oil and particle filters in the compressed air line. The resulting dry, particle and chemical free air was delivered at 60 lb/in^2 to the inhalation exposure system. Bottled air may also be used with appropriate filtration to provide a particle and oil free air supply.

Liquid scintillation counting

Radioactivity was quantified by liquid scintillation counting in a Beckman LS 6000 liquid scintillation spectrometer (Beckman Instruments Inc., Fullerton, CA) using a standard cocktail containing, in 4 L, 2680 mL toluene, 1320 mL Triton X100, 20 g diphenyl oxazole and 2 g bis-(o-methylstyryl)benzene.

Calculations and statistics

Raw particle characterization data were assembled into tables of sample number, time and particle number/mass using FRAMEWORK® and programs written in this laboratory[1]. Various summary tables were then prepared according to the needs of the experimental work.

Calculation of the LCt_{16}, LCt_{50} and LCt_{84} values were done using the established methods of Bliss [9] and Litchfield and Wilcoxon [10] and the newer method of ten Berge [11] as developed by Yee [12]. Both

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1 FRAMEWORK®, originally produced by Ashton Tate, was not supported when Borlan purchased that company. A new version which runs under Windows 95 is now available from Selections & Functions, Inc., Box 505, Scituate, MA 02066.

commercial programs [13, 14] and a newer process developed in the laboratory [12] were used. All procedures utilize PROBIT analysis but the Yee approach employs a newer, more powerful non-linear optimization procedure to fit the PROBIT relationship to the experimental data and so can provide analyses of data which cannot be handled by the earlier Bliss [9] or Litchfield & Wilcoxon [10] methods.

Mean body weights were compared, first using analysis of variance and then, to ascertain which groups were different, using serial two-tailed Student's *t* tests [15].

Description of the exposure system

System configuration

The total inhalation exposure system is modular and may be rearranged to suit the needs of the moment. In the configuration for aerosol exposures described in this report (Fig. 1), the components are arranged in a chain. The supply air, regulated at 40 psi, is split to feed the Lovelace aerosol generator and, passing through a flow controller, to provide dilution air. The diluted (and dried) aerosol is passed though a large bore tubing to the exposure chamber. The exhaust air is pulled from the opposite end of the chamber, through a HEPA filter, a flow monitor and a flow controller. Separate ports from the exposure chamber lead to a Magnehelic gauge to monitor and permit control of the internal pressure in the exposure chamber (should be the same as the surrounding air) and to either an in-line filter or an impactor for taking grab samples of the exposure atmosphere. The trial animals are held on the side of the exposure chamber in a series of common animal holders that readily adapts to mice, rats and guinea pigs. Hamsters could also be accommodated, but have not been used at DRES to date.

Animal holder

The animal holder (Fig. 2) was constructed and machined from Plexiglas® tubing and sheet. The body of the holder for small laboratory animals is a 6.1 cm inside diameter 17 cm long tube. The inside diameter was chosen specifically to provide a close but not tight fit for the polypropylene conical centrifuge tubes used as a disposable sleeve (*vide infra*). A flat plate is attached at one end as the junction with the exposure chamber. The plate has a 4.9 cm hole, centred on the attached tube, which receives the animal nose and guides and positions it in the exposure chamber. At the opposite end there is a threaded closure with an o ring seal and, centred, a plunger attached via a drilled out 0.25 inch Swagelok® union. The plunger has a circular tip sized to match the animal being exposed with a flattened area to allow the tail of the animal to pass the plunger.

For rats and guinea pigs, a sleeve, cut from a 250 mL polypropylene conical centrifuge tube, is used to restrain and position the heads and noses. For mice, a 50 mL conical centrifuge tube is used for the sleeve with spacer washers to keep the tube centred in the holder. The conical tip of each the sleeve is cut off to make a hole fitted to the nose of the species being exposed. When the animals are placed in the holders with the sleeves in place, the conical tip fits into the side ports of the exposure chamber positioning and holding the nose in the exposure atmosphere and preventing the animals from turning or withdrawing their heads.

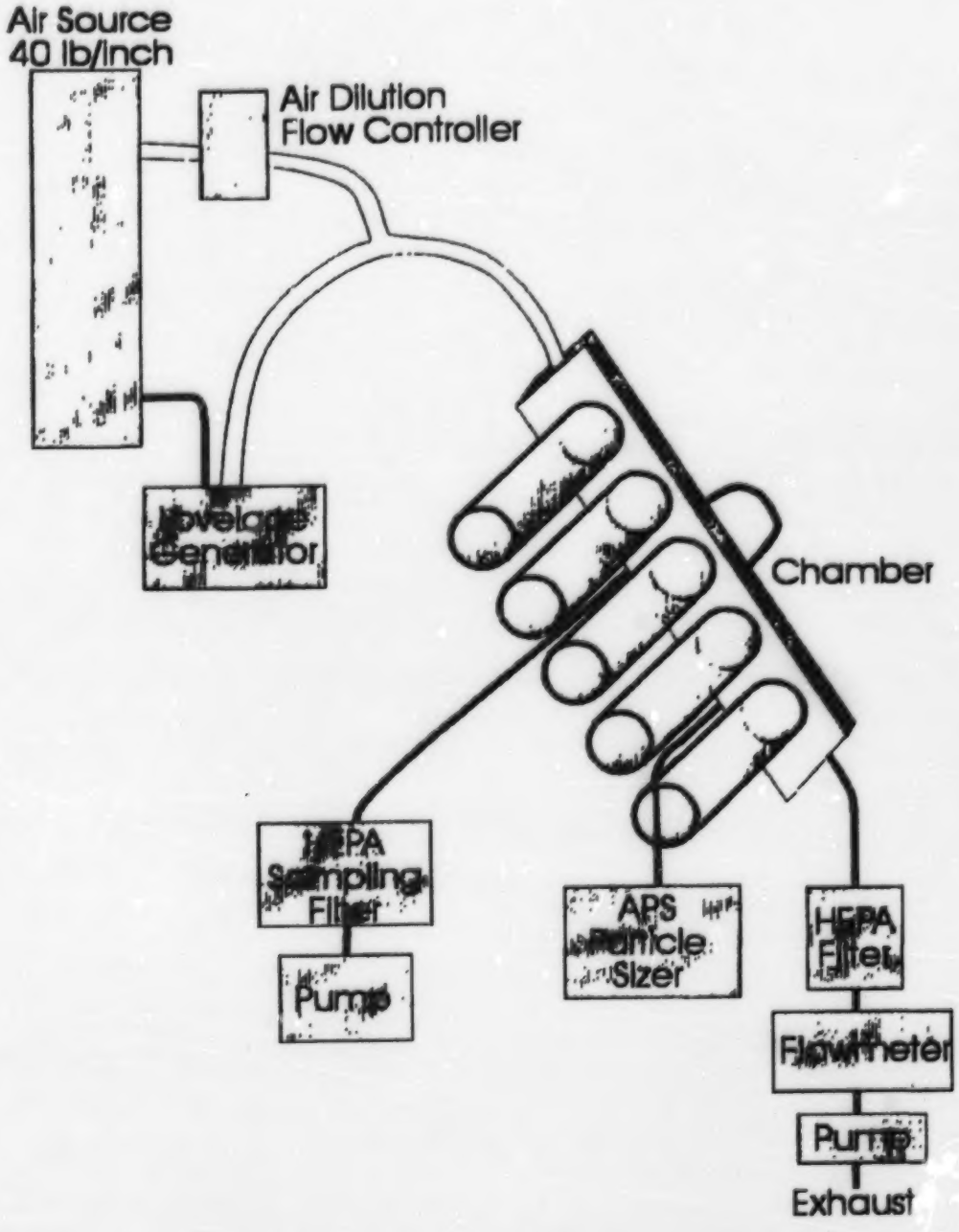


Fig. 1 A diagrammatic view of the aerosol configuration. Air for generation and dilution is supplied at 40 lb/in^2. This results in about 2 L/min of aerosol. Dilution was usually 5 L/min On the sandwich style exposure chamber, the one side was used for animals and the other for sampling and balance measurements The Grab sampling utilized a 1 L/min pump to draw air through the HEPA filter. The APS draws 1 L/min as configured for these studies. The exhaust flow is constantly monitored to ensure that the pressure inside the chamber is the same as in the surrounding air.

Fig. 2 The components of the universal animal holder. The components are, clockwise from bottom left, guinea pig sleeve, rat sleeve, threaded closure fitted with mouse piston, animal holder, closure with rat/guinea pig piston and mouse sleeve with spacer washers in place.

Because the holder is essentially an isolated air space, a respiration monitor can be attached to obtain respiration rates of the exposed animals. A relative, not always quantitative measure of the respiratory volume may also be obtained. A Swagelok® union fitting is mounted on the centre top of each holder to facilitate connection to a respirometer (Columbus Instruments, Cleveland, Ohio). The respiration monitors have not been used to date with this aerosol system. Respiration measurements have been made with these animal holders during exposures to gasses [8].

Exposure chamber

The exposure chamber, of 1 L volume, is a multi-layered or "sandwich" design (Fig. 3). The volume of the chamber may be adjusted somewhat by adding or removing layers of the "sandwich". The test atmosphere enters at one end through an 0.5 in. Swagelok® O-Seal Threaded Male Connector, a port to attach the APS is positioned on the bottom below the 4th animal port[2] and the exhaust is taken from the end opposite the input through a Swagelok® union fitting. The animal holders are clipped onto the sides so the animal noses protrude into the exposure chamber.

2 All indications of position are referenced to the entry port of the chamber

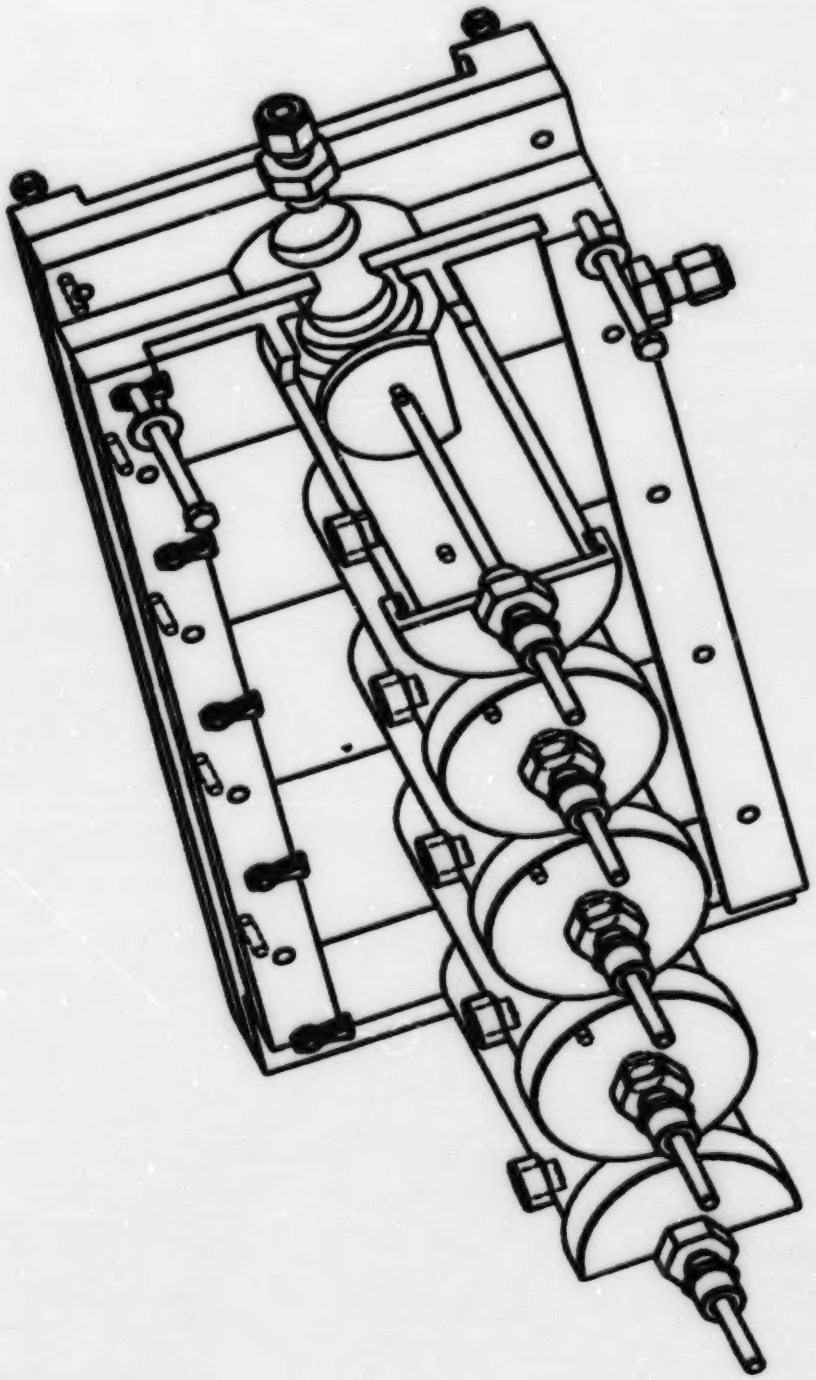


Fig. 3 A cut away view of the small exposure chamber and animal holders

Under routine use in the inhalation toxicity containment suite at DRES, the chamber has 5 animals mounted on one side and the off side ports are closed with rubber stoppers (this is in response to the confined space in the DRES containment facility, all ports may be used for animals when both sides of the chamber are accessible as with gas exposures [8]).

On the opposite side, the third and fourth ports are fitted with one-hole stoppers and glass nipples. The fourth port is used for monitoring the pressure differential between the inside of the chamber and the surrounding air. During exposures, the pressure differential is continuously monitored with a centre-off Magnehelic gauge with a total range of 0.5 mm H_2O. The pressure is controlled manually by adjusting the exhaust flow so that the difference is zero. The centre (3rd) port on the off side is used to take grab samples of the exposure atmosphere.

Generation and Characterization of Aerosols

Source solutions

Source solutions were based on PBS. A minimum of 3 mL was required for normal operation of the aerosol generator for a 10 min exposure. In early experiments, C^{14}-labelled Hb was added to the PBS. In later experiments, C^{14}-sucrose and C^{14}-glucose were used. In all cases, the trace marker was added such that the source solution was ≈20,000 dpm/mL. Measured amounts of toxic material (or solution) were mixed with known amounts of PBS. Samples of the labelled PBS, the original toxic solution and the solution remaining in the generator after exposure were counted and compared to ensure that there were no unexpected changes during the aerosol generation.

Aerosol generation

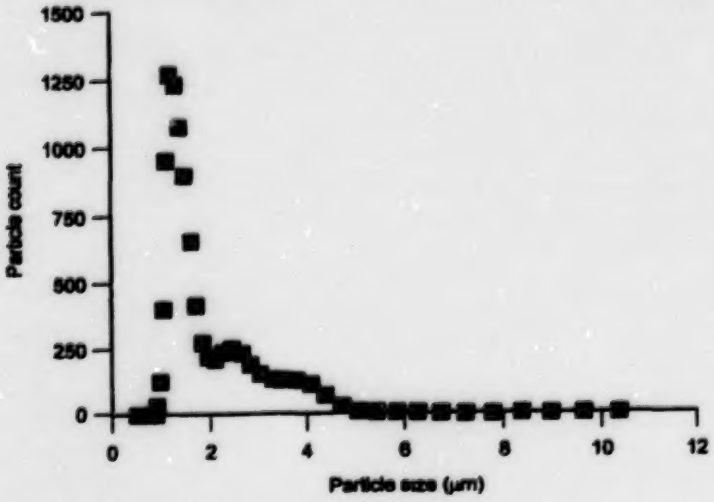
Aerosols for the exposure system were generated using a Lovelace Nebulizer, Model 01-100, Style L009 (Intox Products, Albuquerque, NM) operated at 40 psi input air pressure to provide 2 L/min aerosol. The cap baffle opening was set directly above the spray and beneath the exit tube. The impinger screw is normally set in at three complete revolutions. Working from a starting solution of

Table 2

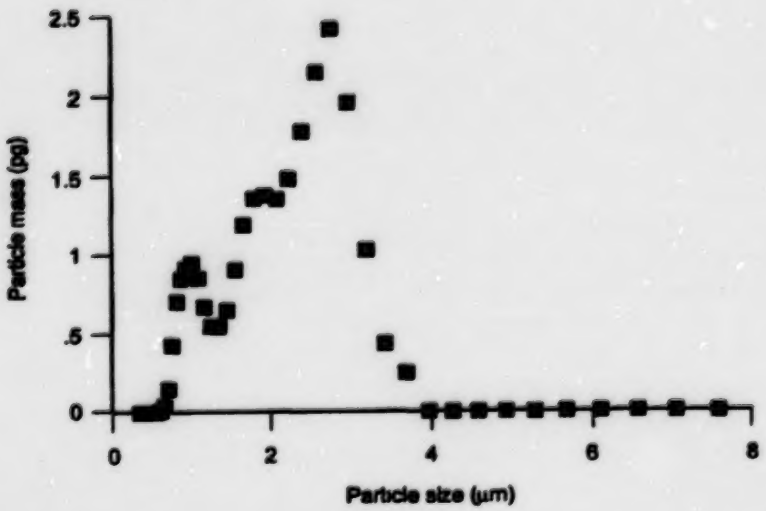
Day to day variation in aerosol generation

Particle size range (μm)	Particle numbers		
	Trial 1	Trial 2	Trial 3
0.50-2.64	23913 ± 585^{4}	23811 ± 609^{9}	23934 ± 447^{16}
2.64-4.37	957 ± 150^{4}	2565 ± 510^{9}	930 ± 447^{16}
4.37-8.35	45 ± 18^{4}	294 ± 69^{9}	231 ± 144^{16}
8.35-14.86	309 ± 24^{4}	225 ± 51^{9}	6 ± 24^{16}

Values are presented as mean $^{N} \pm$ St. Dev.
Data are counts/L * 10^{-3} *ie.* 20,000 = 20,000,000 particles /L



A



B

Fig. 4 Typical particle numbers and mass distributions created by the Lovelace Generator. Chart A shows the particle numbers in the aerosol by particle size. Chart B shows the aerosol mass by particle size. Mass increases as the cube of the particle radius. The original particles are wet and quickly dry during transport to the exposure chamber. The majority of the particles were originally single droplets. A proportion have coalesced into doublet and triplet particles. All particles are within the "respirable range".

Table 3

Comparative particle size and mass spectrum as measured by APS and by Impactor

Particle size (μm)	APS No correction[b]	Particle mass (mg) with added solutes: None		C^{14}-Sucrose[a]	
		APS	Impactor	APS	Impactor
Total	0.620	0.471	0.510	0.477	0.474
<0.176	0.001			0.00	0.008
0.176-0.692	0.03	0.03	0.28	0.07	0.140
0.692-2.54	0.33	0.29	0.22	0.25	0.280
2.54-4.90	0.17	0.12	0.01	0.12	0.026
4.90-9.55	0.09	0.03	0.04	0.03	0.010

[a] Dissolved in PBS.

[b] Values without phantom particle correction [22]. Phantom high mass particles are indicated.

PBS and using a dilution of 5 L/min of breathing air (controlled closely using a TYLAN mass flow controller; RO-28 & FC-260; TYLAN General, Torrance, CA), a consistent, dry [16] aerosol containing ≈24,000,000 NaCl particles/L was delivered to the exposure chamber (Fig. 4, 5). The aerosol, with a geometric mean diameter of 1.072 ± 0.035 μm was consistent day to day (Table 2) and within the respirable range of the test animals [17, 18].

Aerosol Characterization

Aerosol characterizations and particle measurements were made by Laser Doppler Velocimetry [19] using an Aerodynamic Particle Sizer 3300 (APS; TSI Incorporated, St. Paul, MN). The performance of this instrument has been thoroughly characterized for dry [20] and wet aerosols [21]. The APS was modified to sample only through the "inner nozzle" directly into the sizing chamber. "Sheath air" was drawn from the laboratory instead of from the sample stream. This configuration provides for a 1 L/min sampling rate from the exposure chamber to the APS. However, it also makes the instrument
sensitive to differences in air pressure between the sources of sample and sheath air and, for this reason, the difference in pressure between the exposure chamber and the surrounding air was carefully monitored using a Magnehelic gauge and controlled to a near zero value by adjusting the exhaust air flow from the exposure chamber.

The APS was fitted with special software[3] written for DRES (DYCOR Industrial Research, Calgary, Alberta) that, in real time, controls (starts and stops) the APS 3300, reads the particle counts from each measurement (usually at 15 sec intervals), reduces the data from 1024 to 48 ranges between 0.5 and 15 μm, files the results in a continuous ASCII stream file and provides a real-time 3D plot of the particle distribution as the data is collected (Fig. 5). The program restarts the APS as soon as the current measurement data is received by the computer so that the next measurement is in progress

3 A version of this software that runs under Windows® is available from DYCOR Industrial Reasearch.

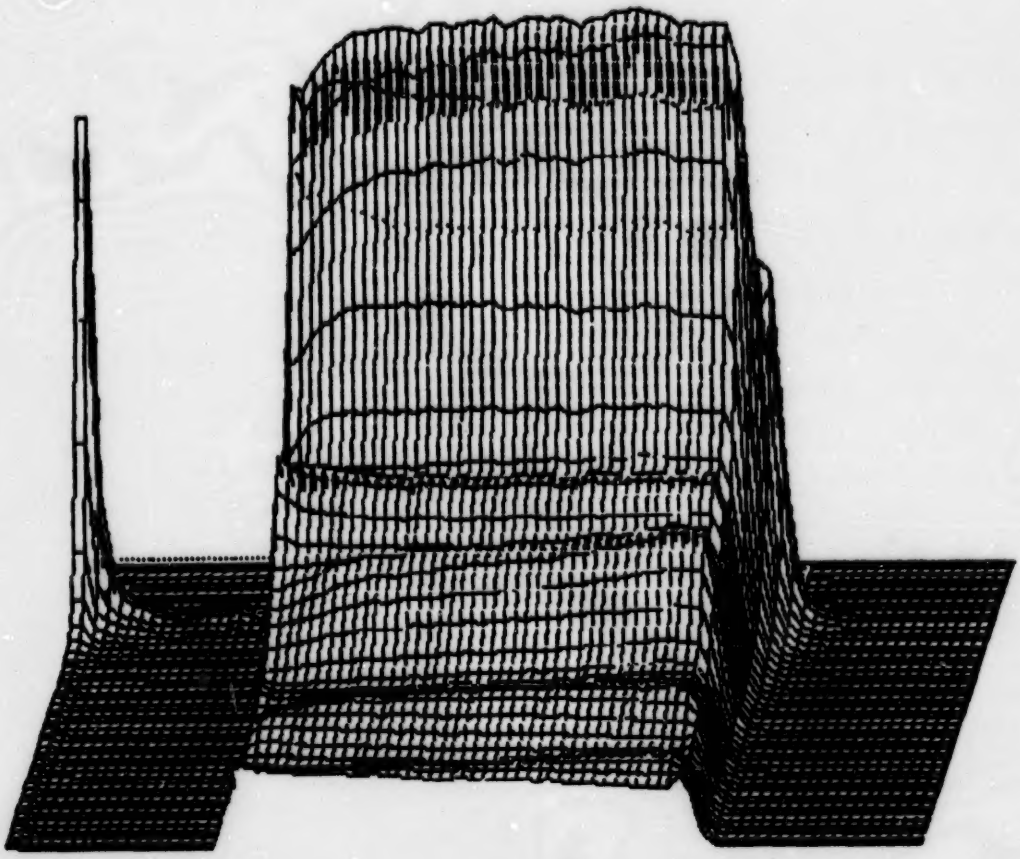


Fig. 5 Particle distribution during a typical exposure experiment. From the left of the diagram, the system initially stabilises and clears any particulate material. Then the aerosol generator is started and the aerosol stabilises. The aerosol is monitored during the exposure and when the aerosol generator is shut off, the aerosol swiftly decays. On the X-axis each line represents a 15 sec interval.

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while the computer processes the current data. In this way, the interval between measurements is normally < 2 sec. Subsequently, a "phantom particle" correction routine [22] is run which significantly reduces the apparent number of larger particles indicated by the APS and improves the correlation between the APS and impactor measurements (Table 3).

The APS was calibrated at regular intervals using an aerosol of latex spheres of known size distribution (1 μm mean mass diameter; Polymer Latex Suspension beads, Duke Scientific Corp., Palo Alto, CA) generated by a TSI Model 3460 Tri-jet aerosol Generator (TSI Inc., St. Paul, MN).

Impactor measurements

As a further check on the character of the aerosol, grab samples (*vide infra*) were collected periodically using an IPP Mercer 7-stage impactor (Intox Products, Albuquerque, NM) with glass fibre filters (#61650; Gelman Sciences Inc., Montreal, PQ) as the collection targets and a controlled 1 L/min flow rate. In this mode, the impactor sampled and sized aerosol particles in the 0 to 15 μm range. The filter papers were preweighed. After sample collection, the filter papers were weighed and, if radio labelled markers were in use, the filters were placed in the bottom of scintillation vials and the radioactivity counted.

Grab sampling of aerosol

Grab samples of the chamber aerosol/air were drawn from the off-side centre port through a 25 mm glass fibre HEPA filter (#61650; Gelman Sciences Inc., Montreal, PQ) in an in-line housing using a 1 L/min pulse pump (AeroVironment Inc., Monrovia, CA). Performance of the pump was checked before and after each exposure with a bubble flowmeter (Gilean Instrument Corp. West Caldwell, NJ). All transfer lines were 0.25 inch ID tygon tubing.

Exhaust air

The exhaust air from the chamber was drawn through an in-line #12144 Capsule HEPA filter (Gelman Sciences Inc., Montreal, PQ) before being vented to the atmosphere. The exhaust flow was continuously monitored using a Digital Flowmeter/Totalizer (Model 545-TOT; Kurz Instruments Inc., Carmel, CA). During routine operation, the exhaust was about 5 L/min with the APS 3300 running. When grab samples were being taken, the exhaust was adjusted to ≈4 L/min. As noted above, the exhaust volume was used to ensure that the pressure differential between the exposure chamber and the surrounding air was always at or very near zero. The exhaust was powered by a Metal Bellows pump (15 L/min capacity; Metal Bellows MB 158 air circulating pump: Metal Bellows Corp., Sharon, MA.) and controlled using a TYLAN mass flow controller.

Calculation of exposure

During the exposure, grab samples (usually two 3 min samples for a 12 min exposure) were taken on HEPA filters using a known air flow rate and sampling time. Radioactivity on the filters was determined by placing the whole filter in a counting vial and the radioactivity counted by liquid scintillation. The dpm/L of exposure atmosphere was calculated using the sampling flow rate, the sampling time and the average dpm on the two filters. As the dpm/weight of toxic material was known, the μg/L air of the toxic substance could be calculated. If the trace of the particle spectrum during the exposure was not smooth, it may be possible to correct the exposure concentration for vagarities in the generation of the aerosol. However, this is a process to be used with extreme caution and usually only in cases where the exposures cannot be repeated.

In several control experiments, it was determined that the difference between the exposure calculated assuming the exposure profile to be a square wave and the exposure calculated by integration of the recorder tracings was less than 5%. Thus, the extra exposure obtained as the concentration falls during the purge cycle and the loss of exposure during the starting, t_{99} period essentially cancelled each other. This relationship assumes that the t_{99} of the system is a small proportion of the total exposure. The recommended ratio of exposure time (T) to t_{99} is T≥13 times t_{99} [23]. If the ratio falls below this limit, serious distortions of the apparent toxicity can result and the assumption in the first sentence is invalid.

To calculate the exposure for aerosol systems, the assumption is made that the radioactivity and the toxic substance in the aerosol are in the same proportions as they are in the source solution. Experience to date would indicate that this is a valid assumption for true solutions of chemicals and proteins. Similarly, radioactive proteins may be used as control substances for protein toxins in solution. In early experiments, bovine haemoglobin, labelled by reacting the -SH groups with C^{14}-iodoacetamide, was used as a marker in the aerosol (*vide infra*) and as the control substance. In later experiments, C^{14}-sucrose and then C^{14}-glucose were used as the marker substance. To date, all source liquids have been true solutions in H_2O. If the source "solution" were to be a suspension or involve mixed solvents with differing vapour pressures, the situation is not clear. Under these conditions, the generation of a consistent aerosol may not occur because one solvent preferentially vaporizes changing the concentration in the source solution or the suspended material may concentrate in the generator. In either case, the calculation described would be compromised and another method of estimating the exposure concentration would be required.

Operating procedure

First, all instruments and air flows are checked and calibrated to ensure proper and controlled operation of the system. Then, the appropriate blank and/or control exposures are run (no animals).

For each animal exposure, the animals, pre-weighed, grouped and placed in caging according to the experimental protocol of the day, are quickly loaded into the holders and mounted on the sides of the exposure chamber. An adequate supply of clean air must be flowing through the chamber. When respiration monitoring is in use, the monitors are connected as soon as all the animals are in place on the exposure chamber.

At time zero (0 min), the clocks and instruments (APS, respiration monitors) are started. The system is run for two min to allow final checks and to record baseline data on all instruments. At time 2 min, the aerosol generator is started and the development of the trial atmosphere is carefully monitored. At the end of the prescribed exposure period, the aerosol generator is stopped by lifting the foot clear of the source solution, air flow through the generator is shut off and the exhaust flow adjusted to maintain a zero balance. When the instruments indicate that the atmosphere in the exposure chamber is essentially cleared of aerosol, the animals are removed and their evaluation begins. For aerosol atmospheres, the clearance is considered complete when the particle count on the APS falls below 4000 particles/L. If the trial substance is known to be extremely toxic, the noses of the animals are wiped before the animals are removed from the holders.

To date at DRES, the usual exposure time for per-acute toxicity studies has been 12 min. The procedure includes taking two, three min grab samples of the aerosol during the exposure. These are usually taken starting at 6 and 9.5 min from the start of the run (4 and 7.5 min from the start of the exposure). When sampling is in progress, the exhaust flow is adjusted to maintain a null balance in the pressures between the inside and outside of the exposure chamber. With care, the grab samples can be taken without alterations appearing in the particle spectra shown by the APS.

In all cases, the timing of events is started (time zero; 0) when the trial substance is introduced. Thus, the first observations concerning the effects upon the animals will come around the 18 min mark (12 min exposure) when the animals are removed from the holders. Unfortunately, no behavioural data can be collected from animals held "nose only" because of the restraint during exposure. Respiration monitoring, in contrast, can continue throughout the exposure and purge periods. The animals are

observed closely for the remainder of the day of exposure, then weighed and examined each morning for the rest of that work week and twice a week thereafter until at least the 21st day. Casualties are examined post-mortem and tissues are examined histologically.

Validation of the procedures

Functional limitations of the system

The exposure system and procedure described will function with soluble biological protein toxins such as ricin and with larger, soluble, non-volatile organic chemicals such as VX [24]. Volatile materials would normally be trialed in a companion system [8]. Non-aqueous systems may be used. A material which has sufficient vapour pressure to sublime in significant amounts from the NaCl aerosol may pose a problem as the result would be a mixed toxicity of vapour and aerosol inhalation. However, for a first estimate of per-acute toxicity, this type of effect may not be of consequence.

None of these materials were affected by or absorbed/adsorbed by the plastics used in the construction of the chambers during the exposure times employed. However, problems may be encountered with materials - *eg.* some organophosphate pesticides - that have higher vapour pressures and may sublime from the NaCl aerosol to absorb into the plastics and then slowly release once the challenge has been removed. These and similar materials are not recommended for use in these Plexiglas® chambers. Steel and glass or an inert plastic should be employed. However, to date no problems of this nature have been encountered.

Most of the work done to date has been with NaCl aerosols from PBS source solutions. The aerosols generated have been essentially constant for each Lovelace generator employed. Minor changes in the 2-4 μm particle numbers have been encountered between individual generators. Between experiments, the foot of the generator should be immersed in distilled water to prevent deposition of salt in or around the air jet orifice. If the generator becomes partially plugged with drying salt, a different particle size distribution will result. Similarly, changing the position of the screw, onto which the air blast impinges, will alter the character of the resulting aerosol.

At DRES, the usual exposure time for this system has been 12 min. With exposure times shorter than 10 min, too great a proportion of the exposure time is in the start-up (t_{99}) and purge periods (with aerosol exposures t_{99} should be assessed on a mass basis). A documented minimum standard for the t_{99}/exposure time relationship has been suggested [23] as total exposure time > 13 times t_{99}. Therefore, in a given exposure system, the lower limit of exposure time is set by the lowest experimental t_{99} value that can be achieved. For longer exposure times, the t_{99} becomes less and less of a factor as the experimental exposures approach the configuration of a square wave. The limiting factors for longer exposure times are the consumption of the source solution and, when that limit is removed, the comfort of the animals and the accumulation of body heat causing high temperatures in the closed animal restrainers. With care, consistent aerosols may be generated for 30 min form a single charge of the Lovelace generator. Longer times may be possible if some mechanism is provided to replenish the source solution on a continuous basis. As of this writing, exposures longer than 30 min have not been made in this system at DRES so the accumulation of body heat has not been a problem.

Relationship between exposure, dosage and particle size

With aerosols, the amount of aerosol retained [17, 18, 25, 26, 27] is a function of particle size, hydration and breathing rate as well as species (physical size of the animals). Particles less than 1 μm diameter are deposited in the airways primarily by diffusion and only 40 to 50% of the inhaled particles are deposited. Larger particles are deposited by impaction and the sites of deposition depend upon the inspiration velocity, the particle size and the structure of the airway. The maximum particle size that will penetrate to the alveoli of a mouse is smaller than that which will penetrate to the rat, dog or human lung. Because the mass of the aerosol particle is a cubic function of the particle diameter, from the same aerosol, the retained dose in the mouse lung is much smaller than that of the rat, guinea pig or human. At the same time, the particles deposited in the upper airways of the mouse include smaller particles that may pass to the lungs of larger species albeit that the cubic mass multiplier may reduce any effects of these smaller particles.

The particle sizes in the saline aerosols described (Fig. 4, Table 2, 3) are such that the majority will penetrate to the lungs of the common laboratory species [17, 26, 27] and deposition of particles in the lungs will be relatively uniform between species. However, small changes in the numbers of 2 - 7 μm particles can mean large changes in the mass of aerosol deposited in the upper airways [28]. This, in turn, means potentially large changes in the apparent dose depending upon the relative absorption rates from the nasopharynx and the rates of expulsion of particulate material from the airways. Thus, the need to monitor the particle size distribution is acute because many factors can alter the size distribution in an aerosol even during the short time of an exposure experiment.

Definition and stabilization of aerosols

The aerosol is dry

Consistency and reproducibility of the toxicologic results requires, in turn, consistent, reproducible and stable aerosol generation and delivery. Aerosols created by the Lovelace generator are wet as they leave the generator. Upon dilution of the aerosol with dry air, the particles dry quickly so that the final aerosol delivered is a dry powder. The drying time of 1 - 8 μm wet aerosol particles is ≈250 msec in 22°C air at 29% *Rh* [16]. Upon leaving the Lovelace Generator, the air will be close to saturated with H_2O. Dilution of the 2 L/min air flow from the generator with 5 L/min dry air reduces the *Rh* from ≈100% to ≈29% and rapidly drys the wet aerosol to salt powder.

Effects of salt on the aerosol

Distilled water as the source solution produces virtually no aerosol (Table 4). The addition of low mg amounts of solute, as would be the case with highly toxic materials, did little more in terms of aerosol concentrations. It was quickly apparent that some form of carrier would be required to create and stabilise the aerosol and make it reproducible with differing experimental conditions and toxic materials. In addition, the solubility and stability of proteins is often limited in distilled water solutions and one aim of this work was to assess toxicity of protein toxins. Further, it is very difficult to obtain salt free protein preparations.

In preliminary experiments, the presence of salts in the source solutions clearly affected the resulting aerosols. Therefore, a number of trials were conducted to assess the effects of varying the salt concentration. As the salt concentration increases both the particle sizes and numbers increase

(Table 4). Because the aim of the work was to estimate toxicity, the aerosol required had to have the particles in the respirable range of the trial animals [17, 25, 26, 27]. The aerosol generated from isotonic saline fulfilled this requirement (Fig.4, 5).

Table 4
Effect of salt concentration on particle size and numbers

Particle size (μm)	Salt concentration (g.100mL) <0.01	0.4	1.0	1.5
0.34-0.81	.61	1811[a]	2680	2496
0.81-1.55	.07	1146	4537	5286
1.55-2.55	.01	97	479	866
2.55-5.0	.01	26	141	196
5.0-7.0	0	6	0	10

[a] Particle numbers are particles/mL air

The potentially damaging effects of salt particles deposited upon the surfaces of the respiratory tract were also considered. As isotonic saline is the solution that "matches" the salt concentration in blood and tissue, PBS *(ie.* buffered isotonic saline) was arbitrarily chosen as a "safe" and convenient form to provide salt in the aerosol. To date no histological damage has been seen which could be attributed to deposition of salt particles within the airways.

Aerosols generated from isotonic saline were very consistent day to day (Table 2). Low levels of co-solutes such as glucose did not affect the resulting aerosol. Other solvents such as polyethylene glycols, however, can drastically alter the particle distribution [29].

Effects of protein concentration

The stability, or lack thereof, of proteins in the aerosol generation process was a serious consideration. The violent physical mixing that occurs in the generator during aerosol generation has the potential to denature delicate proteins. Further, dilute protein solutions tend to be less stable and the dilute source solutions would be expected for highly toxic protein toxins. One method of stabilising protein solutions under similar conditions is to add a "neutral" protein.

The addition of proteins (BSA or Hb) did not affect the aerosol generated from saline (Table 5) until the protein concentrations far exceeded the levels expected for the toxicity studies. At lower levels of BSA (<1 mg/mL), the salt content of the source solution controls the character of the aerosol. Above 1 mg/mL BSA, the protein significantly and progressively increases the particle numbers. Haemoglobin, in contrast, did not affect the character of the aerosol at levels ≤10 mg/mL.

The response of the animals to foreign protein challenge is a major concern when a "foreign" protein is added to the aerosol. No discernable toxic effects have been observed to date in the short term (12 min) experiments performed using either BSA or bovine Hb as stabilizers for protein toxins. Control exposures to protein carrier solutions without toxic material are routine to guard against unexpected responses.

Table 5

Number of respirable particles generated from protein solutions with and without salt.

Solution	Protein concentration (mg/100 mL) 0.08	0.40	1.0	5.0	7.5	10.0
Saline alone	2167±160					
BSA w/o salt	490 ± 155	480 ± 160	494 ± 182	1023 ± 290	1776 ± 463	1942 ± 485
BSA w/ salt	2065 ± 36	2002 ± 26	2050 ± 15	2153 ± 35	2510 ± 45	2771 ± 73
Hb w/o salt	70 ± 36	82 ± 26	92 ± 15	815 ± 16	1182 ± 30	1447 ± 43
Hb w/salt	2335 ± 413	2679 ± 161	2097 ± 267	2095 ± 330	2209 ± 403	2181 ± 444

Values are mean ± standard deviation. All values represent 10 measurements.
Data are given as counts/L * 10^{-4}. 2000 = 20,000,000 particle/L.

Comparison of results from the APS and Impactors

A comparison of the data from the APS and the Impactor (Table 3) shows that the total mass values agree relatively well between the Impactor and the APS after "phantom particle" correction [22]. Using uncorrected APS values produced a high, unacceptable mass value. In this experiment, the proportion of mass in each range of the Impactor indicated by C^{14}-sucrose counting closely matched the gravimetric data.

To confirm that the salt, C^{14}-sucrose and protein were equally distributed in the particles, source solutions containing distilled water, saline, saline-C^{14} sucrose and various concentrations of BSA and Hb in saline C^{14}-sucrose were aerosolized and the aerosol characterized both by APS and Impactors. The filter papers were pre-weighed and weighed after collection to obtain the mass distribution of the aerosol. Then the filters were counted to obtain the distribution of the radioactivity. The APS results were calculated both as particle numbers and mass.

As expected, the distilled water produced little or no aerosol (Table 4). With saline alone and saline-sucrose, the same aerosol was obtained. With BSA concentrations of 0.1 and 0.5 mg/mL and with all concentrations of Hb to 3 mg/mL, the APS particle spectra, the mass from the impactor and the BSA, the heavier particles contained more mass and less radioactivity indicating a disproportionation of the radioactivity on the impactor filters produced comparable values. However, with higher concentrations of source solution in these particles.

Initially, C^{14}-Hb was used as the marker substance. However, following upon the above results and in consideration of the potential problems with "foreign" proteins in the aerosol exposures, it was decided to use C^{14}-sucrose (and later C^{14}-glucose) as the marker chemical in all source solutions when protein stabilisation was not required. The switch to glucose eliminated potential problems with sucrose metabolism. The C^{14}-sucrose came as an ethanol solution. As no ethanol was present in the C^{14}-glucose, the switch also eliminated any potential problems from the ethanol in the aerosols.

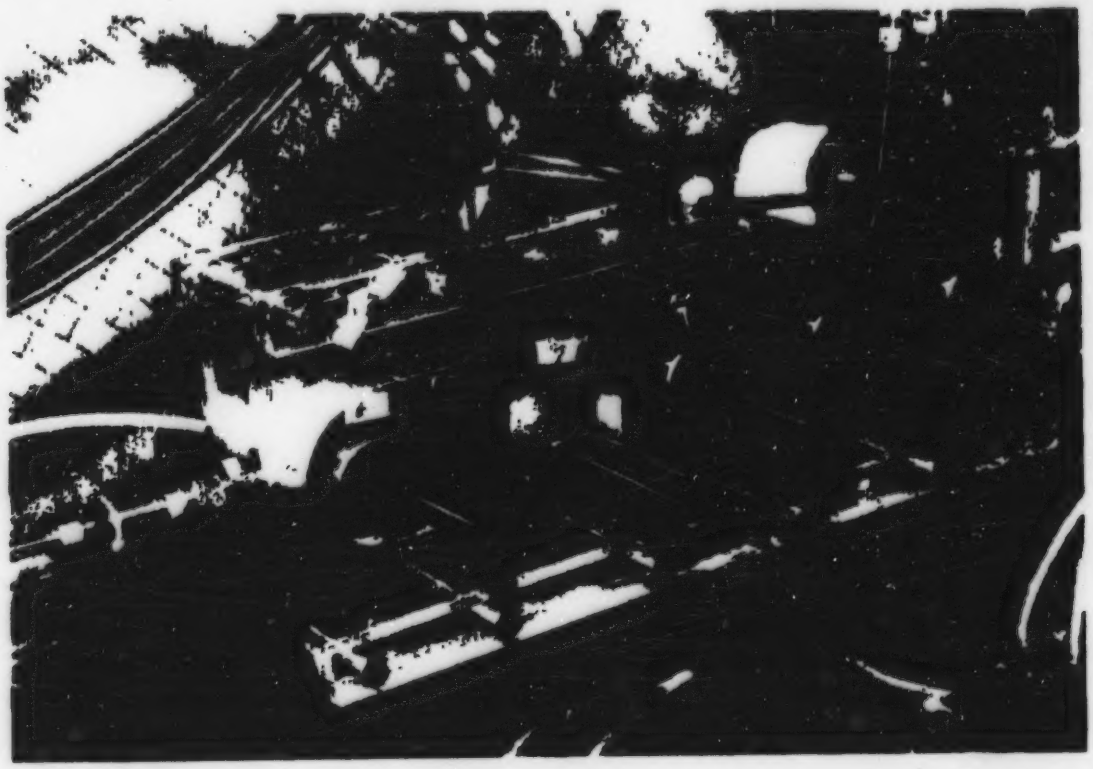

Fig. 6 Pseudo-rats on the inhalation chamber for rebreathing trials. The unit mounted second from the left (with a different stopper) is the "test rat" which is connected to the Harvard ventilation pump. The other "pseudo-rats are constant flow units. Each "rat" draws 200 mL from the chamber and injects 200 mL of clean air. The tips of the two tubes disgorge and sample from a space equivalent to the nostrils of a rat. The flows in these rat substitutes are controlled and constant. The "test rat" has alternating flows to better simulate a breathing animal.

Addressing the rebreathing problem

Studies with other nose only exposure systems [30] have shown that the flow rate of test gas/aerosol to the immediate vicinity of the animals noses was a critical factor in establishing and maintaining the true exposure. As air is taken into the lungs, about 40% of inhaled aerosol particles are deposited in all parts of the airways [25, 26, 27]. Thus, the exhaled air will contain significantly fewer particles of all sizes. In a nose only exposure system, rebreathing of exhaled air from either the animal in question or it's neighbours, will result in a net reduction of aerosol mass inhaled and an unmeasured reduction in the exposure proportional to the amount of "rebreathed" air. To counteract this effect, the flow rates in the chamber system must be and remain above critical levels [30] related to both the minute volume of the species under trial and the volume of the space around the nose available for air exchange. In the chambers described, the exposure of mice was not considered to be of great concern because the mice, breathing at 10 x 25 mL/min from a 1 L volume with a 7 L/min exchange rate, should not affect the particle content of the atmosphere.

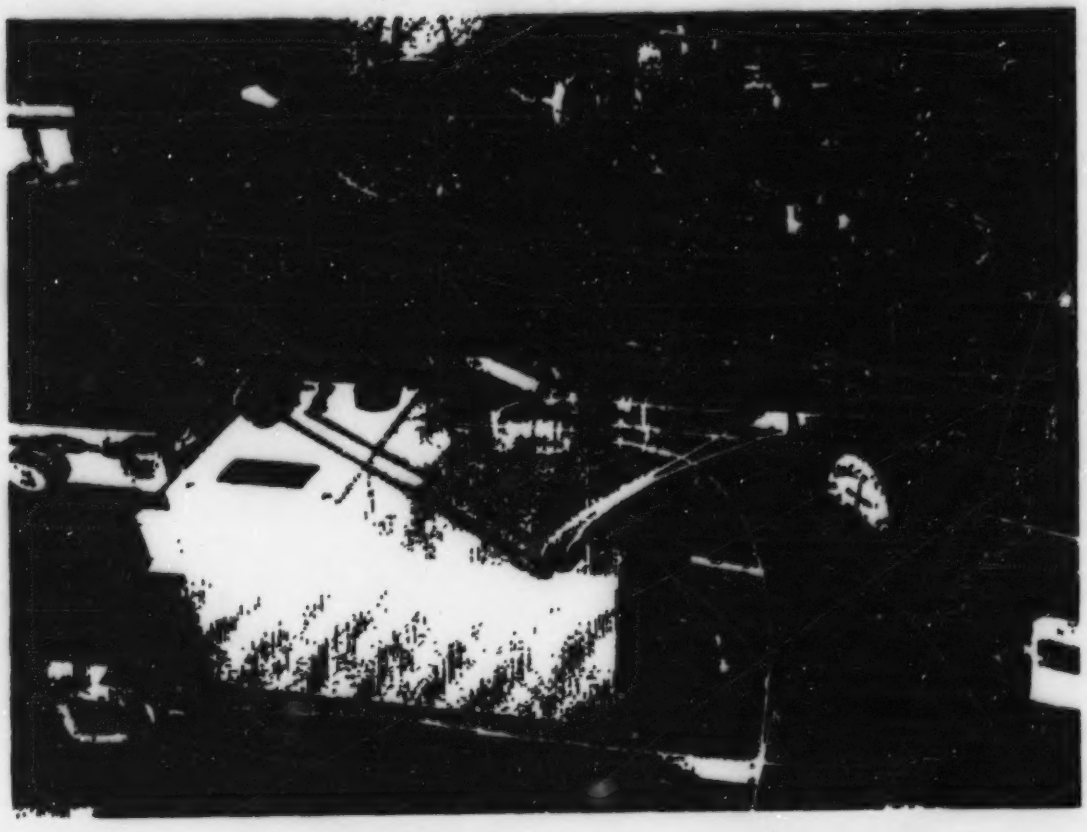

Fig. 7 An overall view of the "rebreathing" trial apparatus The chamber is in the background fitted with 9 "pseudo-rats". The "test rat" and the Harvard ventilation pump are in the foreground.

With rats and guinea pigs, however, there was concern as 5 or 10 animals, each breathing ≈250 mL/min, could retain a significant portion of the aerosol particles and significantly alter the dosage obtained.

The following experiments demonstrated that, with 10 rats breathing 250 mL/min, the variation in exposure between the 5 ports on one side of the exposure chamber did not exceed 5% providing that the input to the chamber was at least 4.5 L/min. For this demonstration, 9 of the 10 animal ports on the exposure chamber were fitted with "pseudo-rats" (Fig. 6) which each continuously withdrew 250 mL/min from the chamber and inserted 250 mL/min of clean air. These "pseudo-rats" plus the "test rat" described below, represent the worst case in which the rats strip the trial substance from the atmosphere and return"clean" air at a rate of 250 mL/min. In the 10th, the test port, the air flows were controlled by a reciprocating small animal ventilation pump (Harvard model 683, Harvard Apparatus Canada, Saint Laurent, PQ.) which, at a fixed reciprocal rate, withdrew an air sample and delivered a corresponding amount of clean air to the chamber. The air sample was fed to a MIRAN Analyzer for estimation of the chemical in the "inhaled" atmosphere. This test port was systematically moved down one side of the chamber to obtain readings from each port.

Table 6

Effect of total flow on exposure measured in each port

Port number	Flow rate L/min			
	2.5	3.5	4.5	5.5
1	97	100	100	100
2	96	97	103	96
3	82	92	100	101
4	63	90	95	97
5	52	84	97	98

Numbers are % of theoretical value

Ports numbered from inlet.

The "pseudo-rats" were constructed as follows. Two, 0.125 inch holes were drilled, 0.5 inch apart, near the centre of rubber stoppers. One-eighth inch O.D. stainless steel tubes, 4 inch long were inserted in each hole and each pair of tubes were bent slightly towards each other so the openings on the inner sides of the stoppers were about 0.125 inch apart, approximating the two nostrils of a rat or guinea pig. For the constant flow "pseudo-rats", one tube in each stopper was connected through a flow controller to a vacuum source and the other was connected via a needle valve controller to a source of clean compressed air. Both the "in" and "out" tubes were set to flow at 250 mL/min. When mounted on the small chamber (Fig. 6, 7), these assemblies represented 9 rats breathing at 250 mL/min. Thus, each "pseudo-rat", with equal in and out air movement, would represent a breathing animal that did not change the total amount of air in the chamber but would strip clean the aerosol from the inspired air. The 10th "test rat" was similarly constructed but the "respiration" was controlled by a Harvard ventilation pump (Harvard Apparatus, Inc., South Natick, Mass.) operating at 250 mL/min(1.67 mL/stroke and 150 strokes/min. The air drawn from the chamber was passed to a MIRAN Analyzer to measure the concentration of butadiene.

A series of experiments were conducted using butadiene as the test chemical and varying the total flow through the exposure chamber from 1 to 8 L/min. In all trials, there were 9 pseudo-rats and one test rat "breathing" from the chamber The effective exposure at each port was estimated, in turn at each total flow rate, by exchanging the constant volume "pseudo-rat" with the "test rat" and measuring the equilibrium concentration reached in each case in the withdrawn air. Throughout these tests, a 1 L/min out-flow was maintained at the port used for APS sampling.

With a total flow of 2.5 L/min, there was a significant decrease in the amount of butadiene recovered as the "test rat" was moved down the chamber away from the gas input (Table 6). When the flow was increased to 3.5 L/min, the difference in exposure measured at ports 1 and 5 was consistently under 15% and, with a flow ≥4.5 L/min, the variation was ≤5%. Starting the grab sample 1 L/min flow did not alter the distributions at the higher flow rates. Of interest was the result that the equilibrium concentration at ports 2 and 4 were consistently lower than at ports 1, 3 and 5. From these results, the minimum total flow of input air to the small chamber was set to 5 L/min. However, in most of the experiments done to date with this system, the total flow was been 7 L/min with the Lovelace generator set to produce 2 L/min and the dilution air set at 5 L/min.

Table 7

Effect of aerosolized ricin on rats

Exposure mg.min/m3	Exposure mg/m³	mort-ality	Body weights at day (g) 0	1	2	3	6	10	13	16	23
Control											
0	0	0	221 ±9	3.8 ±0.9	8.1[a] ±1.1	13[b] ±1.2	17[b] ±1.3	27[b] ±3	41[b] ±5	50[b] ±9	62[b] ±7
Exposed											
2.66	0.222	0/3	215 ±4.2	-1.2[c] ±0.7	-10.8[bc] ±1.8	-12[bc] ±3.7	-5.6[c] ±13	2.2[c] ±11.4	10.6[c] ±15	25 ±23	58[b] ±28
2.76	0.230	0/4	237 ±13	-1.8[c] ±3.0	-9.7[bc] ±5.8	-9.8[ac] ±9.9	-2.6 ±18	9.1 ±28	18 ±29	26 ±28	46[b] ±29
4.37	0.365	2/3	215 ±2.5	-1.9[c] ±2.4	-12[bc] ±1.9	-20	-21	-17	-9.4	11	39
4.40	0.366	1/3	256 ±12	-1.8[d] ±11	-9.3 ±3.7	-7.2	5.5		16	26	50
4.62	0.385	4/4	233 ±13	-4.5[c] ±12.	-14 ±0.5	-21					
4.69	0.391	4/4	220 ±10.3	-4.0[c] ±8.8	-14 ±1.3	-19					
4.78	0.399	2/4	217 ±7.6	-2.5[c] ±0.2	-12[bc] ±0.9	-16[bc] ±2.2	-7.9	-3.6	10.7	28	68
5.68	0.473	4/4	242 ±3.9	-3.0[ac] ±4.2	-13 ±0.7						
5.94	0.495	2/3	293 ±30	-4.4 ±23	-9.8	-13	8.1	23	33	40	59
6.74	0.560	3/3	295 ±19	-4.8[ac] ±2.7	-9.8						
8.45	0.700	4/4	297 ±6.4	-9.0[ac] ±2.4	-15.7						

Body weight values are mean ± standard deviation. If groups contain <3 animals, mean is given.

Control group contained 10 animals

[a] Significant difference from day 0; $P < 0.01$

[b] Significant difference from day 0; $P < 0.05$

[c] Significant difference from Control; $P < 0.01$

[d] Significant difference from Control; $P < 0.05$

Inhalation toxicity of Ricin aerosol

The per-acute toxicity of ricin to male rats was estimated using aerosol generated from source solutions of PBS with various concentrations of ricin and known constant amounts of C^{14}-Hb as the internal marker. Groups of 4 rats were exposed for 12 min to concentrations from 0.01 to 8.0 mg/m³ ricin:PBS aerosol. The total amount of ricin used to obtain this data was <4 mg.

These experiments were the first animal exposures to be done in the chamber system described. In several instances, the animals were not sufficiently restrained (plunger too loose) so that the rats managed to turn their heads back into the animal holders. These animals suffocated and are the reason for several groups being reported as having 3 animals instead of 4. As the operators became more familiar with the equipment, this problem was overcome and, to date, several hundred exposures have been done without further incident. It cannot be emphasized enough that the operation of this type of equipment is close to an art and there is no substitute for experience in the operation of the system and the animal handling involved.

Table 8
Comparison of PROBIT Calculations by three techniques

Parameter [11,12]	Bliss [9]	Litchfield & Wilcoxon [10]	tenBerg/ Yee
LC_{05}	0.237 (0.087:0.293)	-	0.263
LC_{16}	-	0.252 (0.240:0.265)	0.293
LC_{50}	0.346 (0.263:0.391)	0.343 (0.280:0.419)	0.346
LC_{84}	-	0.466 (0.443:0.490)	0.408
LC_{95}	0.504 (0.433:0.961)	-	0.454
Slope	10.05	7.453	13.85
Intercept	9.633[a]	8.466[a]	6.39

The program used for the Bliss calculation [13] provides 1, 5, 95 and 99 LC values. The Litchfield & Wilcoxon program [14] provides 16 and 84 LC values with 95% confidence limits. At this time, the ten Berg/Yee program does not give confidence limits.

[a] These values are artificially raised by 5.0, a "standard" change in use for many years to permit the use of mechanical calculators which did not handle negative numbers. The intercept is seldom used and the slope and 50% values are not affected.

Data from exposures to 0.222 to 0.495 mg/m³ (Table 7) were selected (selected data are marked in Table 7) for PROBIT analysis by the Bliss [9] and Litchfield and Wilcoxon [10] methods both of which limit the number of 0 and 100 per cent values to two of each. All of the data was used for analysis by the tenBerg/Yee technique [12]. The LCt_{50} values obtained were 4.14, 4.11 and 4.15 mg.min/m³, respectively. These values are in good agreement. However, the PROBIT slope values[4] were not so consistent at 10, 7.45 and 13.8, respectively (Table 8). The variation in the calculated slope values

[4] In the author's experience, the differences in slope result from the algorithms used to include 0 and 100% values. The Ten Berg/Yee method uses all values and the result is usually a steeper PROBIT slope.

serves to emphasize, again, the difficulties in predicting both the toxicity from studies with small numbers of animals and the lower toxicity levels (LCt_{01}, LCt_{05}) from PROBIT calculations. The LCt_{50} values are in general agreement with the published values of Griffiths *et al* [31] and Pitt *et al* [32].

Times-to-death were generally 48 - 72 hr. No immediate lethality was observed. However, on day 1 marked effects were present in the body weight data (Table 7) even at the lowest 0.05 mg/m^3 exposure. Recovery was slow and, in many cases, incomplete by the end of the 23 day post exposure observation period. In addition, the large standard deviations in the body weight values after 23 days were indicative of a proportion of the exposed animals that were not "thriving" following their exposure ordeal.

Post mortem examination of the casualties showed many changes detailed in the pathology reports in Appendix A.

Summary

Estimations of inhalation toxicity are a required part of the hazard assessment of potential warfare agents. In many instances, the new materials are either expensive or very difficult to obtain in quantity as well as highly toxic. This report describes, in detail, a system - methods and apparatus - developed and in use at DRES for the assessment of the "nose only" inhalation toxicity of aerosolized protein toxins and larger, non-volatile chemicals. Toxicity values may be estimated, safely, in small laboratory species using small quantities (mg or less) of material. The toxicity values for ricin (LCt_{50} of 4 $mg.min/m^3$ for 12 min exposure), obtained using <4 mg of material, are presented as an example of the utility of the system. Companion reports describe the system as used for estimation of the toxicity of gasses.

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Appendix A

Pathologic reports on casualties by Ricin poisoning

Sample of tissues fixed in formalin were forwarded to VPL Edmonton for evaluation.

The result may be summarized as a general degeneration of the viscera and major organs.

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VPL

DR. CHERNOGRODSKY — V93-3870 to 79

Sex F Species MURINE

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Veterinarian DR. SCHOFIELD

Clinic DEFENCE RESEARCH EST. SUFFIELD

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Collected 09-07-93

Received

Reported 27-07-93 1030

NOSIS

Experimental Ricin Toxicity

MENT

The mice covered by this report are mice 1 to 5 in cage 1A and mice 1 to 5 in cage 2A. There were similar findings in all mice and no differences between those in which the Ricin was administered in Glycerol versus those in which it was administered in saline.

Lesions on the abdominal serosal surfaces appear to be caustic, suggesting that ricin is caustic on contact. The lung changes described, appear to be more a reaction of the lung as an organ to ricin circulating in the bloodstream as it traverses the pulmonary vasculature rather than a true (primary) pneumonia.

The hippocampal lesions are very interesting but I would have liked to see some control animals to be sure that they are due to ricin.

ALL DESCRIPTION

A composite description of the lesions encountered is as follows: the adrenal gland, where present, exhibited multi-focal to locally confluent areas of cortical necrosis. There was no significant degree of inflammation associated with these areas of necrosis. Serosal surfaces of the abdominal cavity and in particular, those of the diaphragm and the intestinal tract exhibited fibrinous exudate within which there were degenerate nuclei as well as nuclear debris. Although specific cell types could not be identified in this debris, it appeared that the debris was composed both of necrotic serosal epithelial cells and small numbers of inflammatory cells. In some animals, there were increased numbers of neutrophils in the intestinal mucosal lamina propria. The lungs of all animals exhibited a diffuse interstitial reaction characterized by large numbers of neutrophils circulating through the alveolar capillaries. This reaction was confined to the alveolar walls and neutrophils were not immigrating into the alveoli or into the airways, although they were present outside the alveolar capillaries in the alveolar wall. In a number of mice, there were large, clear vacuoles associated with the pyramidal cell layer of the hippocampus. These vacuoles were present, both along the edges of the pyramidal cell layers and in the adjacent white matter. These did not appear to involve neuron bodies but were possibly, widely dilated axon sheaths. These vacuoles, however, did not contain any remnants of axons. There was severe lymphoid depletion in the spleen of most of these mice.

Slight variations on this overall description were seen in individual animals:

- Mouse 1 - Cage 1A: this animal had focal agonal hemorrhages present within the lung.

P = Positive

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8
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OWNER | ANIMAL ID / BREED | ACCESSION NUMBER

Age
Sex | Species
Phone

Veterinarian
Clinic
Copies to

Chart Number

Collected
Received
Reported

LOW | NORMAL | HIGH | REFERENCE RANGE / COMMENTS

Mouse 2 - Cage 1A: this animal had focal necrosis on the capsular surface of the liver and extending into the parenchyma immediately underneath the capsular surface. The heart of this animal exhibited agonal sub-endocardial hemorrhage and there were increased number of leucocytes circulating through the veins within the heart.

Mouse 3 - Cage 1A: liver changes in this mouse were similar to those of mouse 2. The lung of this particular mouse exhibited, in addition to the changes described above, peri-bronchial lymphoid nodular development but depletion of these nodules.

Mouse 4 - Cage 1A: consistent with general description above.

Mouse 5 - Cage 1A: agonal sub-endocardial hemorrhage in the heart.

Mouse 1 - Cage 2A: spleen and hippocampus of this mouse were microscopically normal. The pulmonary lesions were present but not nearly as severe as the description above, as there were some normal areas present in the lung of this animal.

Mouse 2 - Cage 2A: a small amount of fibrin was present on the capsule of one kidney of this mouse. The lung of this particular animal was diffusely hemorrhagic throughout and exhibited an intense interstitial neutrophil reaction.

Mouse 3 - Cage 2A: several protein casts were present in renal tubules of this animal. The lung changes in this animal were more similar to those of Mouse 1 - Cage 2A.

Mouse 4 - Cage 2A: some fibrin was present on the capsular surface of the kidney.

Mouse 5 - Cage 2A: there was diffuse interstitial hemorrhage throughout the diaphragm with fibrin on the surface. Sub-epicardial hemorrhage was present within the heart.

P.N. Nation, DVM

S = Sensitive | I = Intermediate | R = Resistant

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4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

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The estimation of military hazard from toxic materials usually requires an estimate of the human toxicity. With expensive, rare and highly toxic substances, only small amounts of substance may be available for testing or safety considerations may restrict the amounts of material that may be used at one time. A functional modular system has been developed for the exposure of mice, rats and guinea pigs to highly toxic liquid, gas or aerosol test materials that requires a minimum of trial material.

The total system is described in this aerosol centred report and in companion reports which describe methods for use with gasses. The aerosol exposure system is operated dynamically in a chamber with a very small volume. Provision is made to continuously monitor the concentration and composition of the aerosol in the test atmospheres and grab samples are taken to establish the exposure concentrations. Respiration of the subjects may be continuously monitored during the exposures but because of the restraint of the animals required by the nose only configuration, observations of behaviour during the exposures are not possible.

The development and validation of this configuration are described and toxicity studies with Ricin are used as examples. The observed nose only inhalation toxicity of Ricin to rats was 4 mg.min/m^3. Times-to-death were generally 24 - 48 hr following significant weight loss Toxicity values from other laboratories range from 2 to 4 mg.min/m^3

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